J. gen. Virol. (1988), **69**, 1949–1955. *Printed in Great Britain* 1949

Key words: *haemorrhagic fever, Korean/Hantaan virus/HFRS*

Conservation of Antigenic Properties and Sequences Encoding the Envelope Proteins of Prototype Hantaan Virus and Two Virus Isolates from Korean Haemorrhagic Fever Patients

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(*Accepted 25 April 1988*)

SUMMARY

Viruses isolated from the blood of two Korean haemorrhagic fever patients were propagated in cell culture and compared to prototype Hantaan virus which was isolated from *Apodemus* mice. The antigenic properties of the human isolates were found to be closely related to Hantaan virus by plaque reduction neutralization, haemagglutination inhibition and fluorescent antibody staining with both polyclonal and monoclonal antibodies. The medium genome segment of each human isolate was sequenced and compared to that of Hantaan virus. Nucleotides comprising the Hantaan virus G1 and G2 envelope protein-coding regions differed from those of the other viruses by only 5·4% and 5·7%. The human isolates differed from one another by 1·6%. The nucleotide differences resulted in predicted amino acid variations of 1·3% to 2·3% among the three viruses, with the majority occurring as conservative substitutions in G1.

INTRODUCTION

Korean haemorrhagic fever (KHF) was first recognized by western physicians as a serious disease threat in 1951, when more than 2400 United Nations troops stationed in Korea developed illness characterized by fever, headache, back and abdominal pain and various haemorrhagic manifestations (Lee, 1982*a*). Although KHF was previously unreported in Korea, similar diseases had been recognized for many years throughout Asia (Lee, 1982*b*). A viral aetiology for the disease was long suspected, but conclusive demonstration of such an agent did not occur until 1976, when antigen reactive with convalescent patient sera was discovered in lung tissues from Korean field mice (*Apodemus agrarius*) (Lee *et al.*, 1978), and after several successive passages in seronegative *Apodemus*, a virus, KHF strain 76-118, was isolated. Conditions for its cell culture propagation were subsequently determined (French *et al.*, 1981), and the virus was renamed Hantaan after a Korean river in the endemic region where the first infected *Apodemus* were trapped (Lee, 1982*b*).

Hantaan and serologically related viruses constitute the newly established *Hantavirus* genus of Bunyaviridae. Viruses in this genus have been associated with numerous clinically similar diseases collectively termed haemorrhagic fever with renal syndrome (HFRS) (WHO, 1982). All hantaviruses currently recognized cause inapparent infections of their rodent hosts and are believed to be transmitted to humans primarily through aerosol exposure to virus contained in the animals' urine, faeces and saliva.

The absence of a good animal model of HFRS has precluded demonstration of a cause and effect relationship of Hantaan virus infection with KHF. Recent isolations of viruses from HFRS patients, however, have afforded the opportunity to compare and contrast a virus

isolated from a natural rodent host to those associated with clinical disease in humans. These data provide important and useful insights into the antigenic and genetic stability of the virus over time, and confirm the aetiological role of Hantaan virus in KHF.

METHODS

Viruses and cells. The isolation and cell culture adaptation of Hantaan virus (76-118) has been described (Lee *et al.*, 1978, French *et al.*, 1981). Propagation and assay of Hantaan virus in Vero E6 cells (ATCC C1008) was as previously reported (Schmaljohn *et al.*, 1983).

The Lee isolate (77-137) was obtained by inoculation of viraemic blood from a KHF patient into seronegative *A. agrarius* (Lee *et al.*, 1981*b*). After three *Apodemus* passages, lung and kidney specimens were homogenized and incubated with A549 (ATCC CCL185) cells. Cultures were monitored for the presence of viral antigen by indirect immunofluorescence and, after several blind passages, the isolate could be readily propagated in cell culture. Because the virus inoculum was contaminated with reovirus, the Lee isolate was passaged four times in Vero E6 cells in the presence of reovirus antisera (types 1, 2 and 3). Two additional passages without reovirus antisera were performed, and the absence of extraneous viruses and mycoplasma was confirmed by both culture and electron microscopic examination.

The HoJo isolate (ROK 83 61) was obtained without prior passage in *Apodemus* by inoculation of Vero E6 cell cultures with viraemic blood from a KHF patient. Cell cultures were incubated at 37 °C for 14 days, after which cells were examined for the presence of viral antigen by indirect immunofluorescence. Samples found to contain viral antigen were cocultivated with an equal number of uninfected Vero E6 cells and incubated at 37 °C for 14 days. Cocultivation was repeated twice more at 14 day intervals, and the final cell supernatant was assayed for infectious virus by plaque formation on Vero E6 cells.

Immune animal sera. The preparation of hantavirus-immune sera in experimentally infected rats has been described (Schmaljohn *et al.*, 1985). Immune rabbit sera were generated by a single intramuscular inoculation of at least two New Zealand white rabbits, each with infectious, cell culture-propagated Hantaan, Puumala, Prospect Hill or Seoul urban rat viruses. Rabbit sera were tested at weekly intervals for the presence of virus-neutralizing antibody, and high-titre sera were pooled as reference antisera for each of the four viruses. One of the monoclonal antibodies (MAbs) used in these studies (HCO2) was obtained from Dr J. McCormick (Centers for Disease Control, Atlanta, Ga., U.S.A.). Hybridomas were prepared using the spleens of mice immunized with inactivated Hantaan virus (J. McCormick, personal communication). For production of additional MAbs, 6- to 8-week-old, female BALB/c mice were immunized intramuscularly with preparations of Hantaan virus envelope glycoproteins in Freund's complete adjuvant. Twenty days later, the same antigen in Freund's incomplete adjuvant was administered intramuscularly. The final immunization consisted of an intraperitoneal injection of the same antigen without adjuvant 18 days after the second immunization. Four days later, spleens were harvested and cells were fused to SP2/0-Ag-14 partners according to standard procedures (Early & Osterling, 1985). Ascitic fluids were prepared according to the method of Brodeur *et al.* (1984). Antibody specificity was determined by immunoprecipitation of radiolabelled viral proteins as previously described (Schmaljohn *et al.*, 1986). Plaque reduction neutralization reaction conditions have been described (Schmaljohn *et al.*, 1985).

Sequence analysis. Oligonucleotides corresponding to the following nucleotides with respect to 5′ viral complementary sense RNA of Hantaan virus were synthesized on an Applied Biosystems model 541 DNA synthesizer: 23 to 42, 101 to 120, 223 to 242, 318 to 337, 494 to 513, 535 to 554, 576 to 595, 611 to 630, 732 to 751, 805 to 824, 1079 to 1099, 1113 to 1132, 1253 to 1273, 1395 to 1414, 1502 to 1522, 1689 to 1708, 1698 to 1716 , 1931 to 1950, 2067 to 2086, 2216 to 2235, 2501 to 2520, 2629 to 2647, 2764 to 2783, 3056 to 3075, 3070 to 3088, 3161 to 3180, 3258 to 3277 and 3375 to 3394. Additional primers required to read ambiguous sequences were prepared with sequence data determined for HoJo. The primers were annealed to purified virion RNA and dideoxynucleotide chain termination sequence reactions were performed essentially as described (Seif *et al.*, 1985). Briefly, 5 to 10 μg of RNA and 0·5 μg of oligonucleotide primer were denatured with 50 mM-methylmercuric hydroxide at room temperature for 10 min in a reaction volume of 10 μl. Two μl of 350 mM-2-mercaptoethanol was added and incubation continued for 5 to 10 min. The RNA–primer solution was then added to a reaction mixture containing 4 μl 1 M-Tris–HCl pH 8·3, 6 μl 1 M-KCl, 2 μl 0·2 M-$MgCl_2$, 2 μl each of 1 mM-dATP, dCTP, dGTP and dTTP, 4 μl [α-^{32}P]dATP (3000 Ci/mmol; Amersham) and 2 to 3 μl avian myeloblastosis virus reverse transcriptase (Life Sciences, St Petersburg, Fla., U.S.A.). Nine μl of this reaction mixture was added to each of four tubes containing 2 μl 50 μM-dideoxy ATP, GTP, CTP or TTP, and tubes were incubated at 43 °C for 15 min. Reactions were terminated by the addition of 5 μl of loading buffer (95% formamide, 10 mM-EDTA). Samples were boiled for 3 min prior to gel electrophoresis.

RESULTS

Serological comparisons

At least four antigenic groups of hantaviruses can be differentiated by plaque reduction neutralization tests (PRNT) (Schmaljohn *et al.*, 1985). Immune sera to viruses representative of

Table 1. *Plaque reduction neutralization of Hantaan, HoJo and Lee viruses with polyclonal and monoclonal antibodies*

	Virus		
	Hantaan	HoJo	Lee
Immune serum*			
Rabbit anti-Hantaan	2560	1280	2560
-HoJo	1280	1280	1280
-Prospect Hill†	2560	2560	2560
-Puumala†	10	10	10
-Seoul†	160	160	320
Rat anti-Lee	5120	2560	5120
-Hantaan	5120	2560	5120
Human anti-KHF convalescent	1280	640	640
-Hantaan	640	640	640
Monoclonal antibody‡			
Anti-G2			
HCO2	40 960	40 960	40 960
11E10	320	320	320
16E6	320	160	320
Anti-G1			
2D5	1280	1280	1280
3D5	5120	10 240	5120
16D2	2560	1280	5120

* Rabbit and rat sera from experimentally infected animals and human sera from a KHF patient or from a subclinical laboratory infection with Hantaan virus were reacted with each virus as described in Methods. Titres are the reciprocal of the dilution resulting in greater than 80% reduction in viral plaques.

† Homologous titres for Prospect Hill, Puumala and Seoul viruses were 2560, 1280 and 5120, respectively.

‡ Mouse monoclonal ascitic fluid was prepared with each of the indicated hybridomas and reacted with viruses as described in Methods.

each group (Hantaan, Prospect Hill, Puumala and Seoul) were used in a PRNT to compare two viruses, HoJo and Lee which were isolated from KHF patients, with prototype Hantaan virus. Immune animal sera were prepared by experimental infection of rabbits or rats as described in Methods. Human sera were obtained from a convalescent KHF patient and from a laboratory worker asymptomatically infected with Hantaan virus. None of the sera produced more than twofold differences in neutralization titres with the three viruses (Table 1). As previously demonstrated with rat immune sera (Schmaljohn *et al.*, 1985), rabbit sera to Puumala virus, the aetiological agent of nephropathia epidemica, had a very low neutralizing titre with Hantaan virus and, similarly, poorly neutralized HoJo and Lee. Immune rabbit sera generated to Seoul virus produced low PRNT titres with Hantaan virus, despite a high (1:5120) homologous neutralization titre; rabbit sera generated to Prospect Hill virus produced PRNT titres with Hantaan virus equivalent to the homologous titre. Therefore, each serum examined produced consistent PRNT titres with Hantaan, HoJo and Lee viruses, and failed to distinguish between them.

Plaque reduction by six Hantaan virus-specific, neutralizing MAbs also failed to differentiate prototype Hantaan from HoJo and Lee viruses. Three G2-specific and three G1-specific Hantaan MAbs produced PRNT titres with HoJo and Lee viruses that did not differ by more than twofold from those obtained with Hantaan virus (Table 1). The serological similarities of these three viruses were confirmed by haemagglutination inhibition and indirect immunofluorescence with a panel of 23 Hantaan virus MAbs. Each of six G1-specific MAbs reacted indistinguishably with the three viruses, and only one of 17 G2-specific MAbs demonstrated reduced reactivity with HoJo and Lee as compared to Hantaan (data not shown).

Table 2. *Nucleotide sequence differences in the G1 and G2 coding regions of the M genome segments of Hantaan, HoJo and Lee viruses*

	No.	Hantaan	HoJo	Lee	No.	Hantaan	HoJo	Lee	No.	Hantaan	HoJo	Lee	No.	Hantaan	HoJo	Lee
G1	106	C	T	C	862	C	T	T	1543	T	A*	C	2401	G	C	A
	133	T	C	C	877	G	A	A	1558	A	G	G	2413	T	C	C
	142	T	C	C	883	T	C	C	1559	A	G*	A	2428	T	C	C
	145	T	C	C	892	G	A	A	1562	G	A*	A*	2443	C	T	T
	154	C	T	T	906	T	C*	C*	1588	T	C	C	2452	A	A	G
	166	T	C	C	911	T	A*	A*	1600	T	C	C	2464	G	A	A
	172	A	G	G	916	T	C	C	1609	T	C	C	2467	T	C	C
	185	G	A*	A*	925	C	T	T	1684	C	T	C	2536	C	T	T
	196	C	T	T	952	T	C	C	1711	A	G	G	2575	C	A	A
	209	A	C*	C*	977	T	C	C	1714	A	A	G	2587	T	C	C
	229	T	C	C	987	A	T*	T*	1729	A	G	G	2593	G	A	A
	231	A	G*	G*	988	T	C*	C*	1741	A	G	G	2627	T	C*	T
	250	G	A	A	1004	T	T	G	1750	T	C	C	2629	T	C*	C
	253	G	A	A	1024	A	G	G	1756	C	T	T	2650	T	T	C
	316	T	C	C	1042	T	T	C	1759	T	C	C	2662	T	C	C
	321	A	A	C*	1045	G	A	A	1798	T	C	C	2671	T	C	C
	340	C	T	T	1090	T	T	C	1807	G	A	A	2686	T	T	C
	373	A	G	G	1100	A	G*	G*	1828	A	G	G	2704	T	C	C
	382	A	G	G	1117	A	G*	G*	1855	T	C	C	2743	C	T	T
	388	G	A	A	1136	T	G*	T	1864	A	G	G	2761	T	C	C
	397	A	T	T	1142	G	C*	G	1867	T	C	C	2791	G	A	A
	403	T	C	C	1150	C	T	T	1870	T	C	C	2797	G	A	A
	409	G	A	A	1153	A	T*	G	1888	G	T	T	2812	C	T	T
	416	G	A*	A*	1155	C	G*	C	1894	A	G	G	2842	C	T	T
	427	C	T	T	1157	G	A*	A*	1915	C	T	T	2884	A	G	G
	433	G	A	A	1165	T	C	C	1951	T	T	A	2890	C	T	T
	436	T	A	A	1167	G	T*	G	1955	T	C	T	2902	T	C	C
	445	C	C	T	1174	T	A	A	G2	1963	C	T	T	2911	G	A	A
	451	C	T	T	1180	C	T	T		1969	G	A	G	2914	G	A	A
	460	A	A	T	1192	T	A	T		1999	A	G	G	2920	T	C	C
	493	C	T	C	1210	A	G	G		2008	C	C	T	2974	T	C	C
	496	T	C	T	1245	C	C	A*		2029	G	A	A	3001	G	A	A
	544	G	A	A	1246	T	C	C		2039	G	A*	G	3007	C	C	T
	558	G	C*	C*	1267	A	G	G		2050	T	C	C	3028	A	A	G
	559	T	C*	C*	1309	G	A	A		2062	G	A	A	3088	T	C	T
	562	C	T	T	1324	C	T	C		2083	C	T	T	3109	G	A	A
	565	T	C	C	1325	A	G*	G*		2104	T	C	T	3118	A	A	G
	580	G	A	G	1333	T	C	C		2111	T	C	C	3142	G	A	A
	589	G	A	A	1348	C	C	T		2126	G	X†	G	3171	G	A*	A*
	595	C	T	T	1357	G	A	A		2127	A	X	A	3196	A	G	A
	610	A	G	G	1369	A	G	G		2128	G	X	A	3198	A	C*	A
	619	C	T	T	1379	C	T	T		2146	C	T	T	3201	T	A*	T
	631	G	A	G	1393	G	A	A		2170	G	A	A	3229	T	A	A
	640	C	C	T	1402	T	C	C		2182	T	G	A	3238	A	C	C
	649	T	C	C	1420	C	T	T		2188	G	A	G	3256	A	A	G*
	667	A	G	G	1429	A	G	G		2195	C	T	C	3265	T	C	C
	670	T	C	C	1444	T	C	C		2296	T	C	C	3270	T	A*	A*
	679	T	C	C	1456	T	A	T		2305	A	G	G	3283	G	A	A
	766	A	G	A	1460	T	T	C		2323	G	A	A	3373	C	T	T
	769	A	T	G	1474	G	A	A		2353	T	C	T	3379	T	C	C
	778	T	C	C	1519	C	T	T		2389	C	T	T	3397	C	A	A
	850	T	C	C	1541	A	G*	A		2395	T	C	C	3409	A	G	G
										2397	A	G*	G*	3442	A	G	G

* Nucleotides that resulted in an amino acid change with respect to Hantaan virus. Numbers correspond to those of Hantaan virus beginning at the 5′ end of the viral-complementary sense RNA.

† X at positions 2126 to 2128 of HoJo virus denotes a deleted codon.

Nucleotide sequence analysis

Like those of other Bunyaviridae, the medium (M) genome segment of Hantaan virus encodes the viral envelope glycoproteins G1 and G2 (Schmaljohn *et al.*, 1987). Antibodies to these proteins are presumed to play an important role in virus neutralization and immunity. Previous comparison of the RNA of Hantaan and Lee viruses by two-dimensional oligonucleotide mapping demonstrated genetic diversity in each of the three viral genome segments (Schmaljohn & Dalrymple, 1984). To examine the extent of that diversity, the gene regions encoding the envelope glycoproteins of HoJo and Lee viruses were compared to those of Hantaan virus.

The nucleotide sequences of the M genome segments of HoJo and Lee were determined by primer extension and dideoxy chain termination with synthetic oligonucleotide primers and

Table 3. *Variation in the predicted amino acid sequences of the G1 and G2 envelope proteins of Hantaan, HoJo and Lee viruses*

	G1 amino acids				G2 amino acids		
No.*	Hantaan	HoJo	Lee	No.*	Hantaan	HoJo	Lee
32	V	M	M	650	V	I	V
40	M	L	L	679	E	X†	E
47	N	S‡	S‡	769	Q	R‡	R‡
77	N	N	T‡	846	C	R	C
109	V	I	I	1027	R	K	K
156	S	T	T	1036	Q	P‡	Q
272	I	T‡	T‡	1037	I	N‡	I
274	S	T	T	1055	I	I	M
299	Y	F	F	1060	V	E‡	E‡
305	Y	Y	D‡				
337	S	G‡	G‡				
342	I	M	M				
349	L	V	L				
351	G	R‡	G				
354	E	D	E				
355	A	G	A				
356	V	I	I				
359	C	F‡	C				
385	S	S	Y‡				
412	M	V	V				
484	I	V	I				
490	T	A‡	T				
491	V	I	I				

* Amino acids are numbered from the amino-terminal amino acid of Hantaan virus G1 (Schmaljohn *et al.*, 1987).

† X indicates a deleted amino acid.

‡ Non-conservative amino acid differences from Hantaan virus according to the following criteria for conservative substitutions: R=K, S=T, D=E, Q=N, V=L=I=M, A=G, A=V, Y=F.

virion RNA templates. With respect to the coding region for Hantaan virus G1 and G2 (nucleotides 92 to 3445), 180 nucleotide differences (5·4%) in Lee virus and 190 (5·7%) in HoJo virus were detected (Table 2). The majority of the nucleotide differences were silent substitutions, i.e. only 31 HoJo virus nucleotides (0·9%) and 21 Lee virus nucleotides (0·6%) contributed to changes in the predicted amino acid sequence of the polyprotein encoded in Hantaan virus M RNA. Many of the nucleotide variations observed were common to HoJo and Lee viruses, which had 53 nucleotide differences (1·6%) from each other, of which 15 (0·4%) contributed to predicted amino acid differences.

In addition to individual nucleotide variations, sequence analysis of the G2-coding region of HoJo virus revealed the deletion of three bases (one codon) at nucleotide positions 2126 to 2128.

Comparison of the predicted amino acids comprising G1 and G2 of Hantaan, HoJo and Lee viruses

Comparison of the predicted amino acid sequences of the envelope proteins of the three viruses revealed differences with respect to Hantaan virus of 20 (1·7%) and 27 amino acids (2·3%) for Lee and HoJo viruses respectively (Table 3). The two human isolates shared all but 15 (1·3%) of their predicted G1 and G2 amino acids. None of the amino acid differences occurred at any of the potential seven asparagine-linked glycosylation sites or at the amino or carboxy termini of G1 or G2 (Schmaljohn *et al.*, 1987). Of the amino acids that differed among the isolates, most were conservative variations with less than 1% of the total amino acids appearing as non-conservative differences. Although more amino acid differences were observed in the predicted G1 proteins than in the G2 proteins of these viruses, the proportion of non-conservative changes was almost the same (0·4 to 0·9%). Consequently, hydropathicity profiles of both of the M segment gene products of the three viruses were nearly indistinguishable (not shown).

DISCUSSION

We have compared the antigenic properties and gene sequences of two virus isolates from acutely infected KHF patients to those of prototype Hantaan virus which was originally isolated from *Apodemus*. These studies were intended (i) to confirm the aetiology of KHF, (ii) to delineate antigenic and genetic variations of viruses isolated at different times from a persistently infected natural host compared to isolates from an acutely infected dead-end host and (iii) to gain insight into the advisability of using prototype Hantaan virus strain 76–118 for the development of an HFRS vaccine.

Antigenic comparisons by haemagglutination inhibition, immunofluorescence and PRNT revealed little difference between the two human isolates and Hantaan virus. Epitope variation was suggested only by the reduced reactivity of a single Hantaan virus-specific, anti-G2 MAb with HoJo and Lee viruses. This MAb did not neutralize any of the three viruses; the significance of this is currently not known. None of the immune sera or MAbs used in these comparisons distinguished HoJo virus from Lee virus.

Sequence analysis of the viral M genome segments confirmed the similarity of Hantaan, HoJo and Lee viruses and suggested a remarkable conservation of their envelope glycoproteins. These data suggest stability of the genes encoding the viral proteins that are most likely to encounter natural immune modulation and selection, i.e. the viral envelope proteins. This stability, not only in dissimilarly affected hosts (acute or persistent infection) but also over time (6 years), may indicate an absence of immunological pressures that would cause viral mutation or, conversely, pressures which continually select for a certain virus population and prevent the emergence of variants. It has been shown that, although experimentally infected *Apodemus* produce Hantaan virus-neutralizing antibodies, infectious virus continues to be excreted in the animals' urine, faeces and saliva for at least 1 year after infection, with no apparent deleterious effect on the rodents (Lee *et al.*, 1981*a*, *b*). It is not known at present how the virus escapes neutralization, or even whether neutralization is the primary mode of recovery from virus infection. Whatever selective pressures exist in nature, it is clear from our studies that the *Apodemus* isolate, Hantaan virus, and the two isolates from KHF patients are nearly identical, and perhaps should be considered strains of the same virus.

Our results suggest that a vaccine developed to Hantaan virus, whether by classical methods or recombinant DNA technology, should be equally reactive with viruses known to cause KHF. Ideally, a vaccine targeted at KHF should also be able to protect against other HFRS illnesses. With the information currently available, it is impossible to predict the success of such a vaccine with HFRS in general. Studies similar to those described here, with viruses in the other three hantavirus antigenic groups, would help to define conserved gene regions among all hantaviruses, thus enabling the development of vaccines or diagnostic reagents which are cross-reactive among many different hantaviruses.

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(*Received 16 February 1988*)